Thymol as natural antioxidant additive for poultry feed: oxidative stability improvement

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ABSTRACT Antioxidant are regularly included in poultry feed as protection from deterioration during storage. Recently the interest for the use of natural phytochemicals in animal diets has been increased. Thymol (THY) has been proven to be an effective antioxidant for extending broiler meat quality during storage with similar action to the widely used butylated hydroxytoluene (BHT). This study evaluates whether THY can also have a protective effect on the feed mash by assessing its antioxidant potential and related changes in fatty acid (FA) balance. Feed mash was assigned to 1 of 4 treatments, control (CON, no additive), vehicle (VEH, ethanol 96%), BHT (400 mg BHT /kg feed) and THY (400 mg THY /kg feed). Three replicates of each treatment were taken after 0, 30, and 60 d of storage at room temperature (23 ± 3°C) and relative humidity (40 ± 5%). Peroxide value (PV), titratable acidity (TA) and FA relative composition were determined. As expected, there were no treatment effects on those variables at 0 d of storage. However, higher PV values were detected in the CON and VEH groups after 30 and 60 d of storage in comparison to the THY and BHT treated samples (CON = VEH > THY = BHT). While a slight increase was also observed in TA through storage time, no particular treatment effects were detected. Relative FA composition changed with storage time only in the CON and VEH group which had a decrease in polyunsaturated fatty acids and an increase in saturated FA. No changes were detected in the Thy and BHT treated feeds. The results suggest a similar THY and BHT protective effect on feed mash lipid oxidation. Thus, THY could be considered as a useful natural alternative to help sustain quality of poultry feed.

Key words: essential oil, diet, poultry, thymol, PUFA, peroxide value

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INTRODUCTION

Many natural and synthetic antioxidants have been shown to decrease or delay the undesirable evidence of oxidation progress. This became relevant considering that consumer concerns relative to the quality of animal products has greatly increased during the past decades (Min and Ahn, 2005; Shah et al., 2014) and that quality and healthfulness were two of the most important factors influencing the choice of the consumer for foods (Lennernäs et al., 1997). There are many studies showing that feeding poultry with antioxidant compounds added into the diets containing antioxidants will improve the oxidative stability of animal products (Botsoglou et al., 2003; Lee et al., 2004; Govaris et al., 2007; Smet et al., 2008; Luna et al., 2010; Karre et al., 2013; Haselmeyer et al., 2015). This could subsequently enhance the value of poultry products since poultry meat is especially prone to oxidative deterioration due to its high content of polyunsaturated fatty acids (**PUFA**; Igene and Pearson, 1979), and that oxidation of lipids is one of the most important factors that impact on quality loss of animal foods. All these potential benefits coupled with changes in some countries regulations on the use of synthetic compounds in animal nutrition have stimulated the interest for further research in the use and potential effects of phytochemicals in the diets of farmed animals. Furthermore, studies on butylated hydroxytoluene (**BHT**), a widely used synthetic antioxidant compound, have shown potential toxicity and adverse effects (Olsen et al., 1986; Madhavi et al., 1996; Carocho and Ferreira, 2013). It has been demonstrated that herbs and spices are compounds that, when included into animal diets, are generally effective to help sustain good health, performance, welfare of the

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animals, and the oxidative stability of their products (Hashemi and Davoodi, 2010; Amad et al., 2011; Borazjanizadeh et al., 2011; Alali et al., 2013; Labaque et al., 2013; Shah et al., 2014; Chen et al., 2016; Hafeez et al., 2016).

Oregano is an aromatic plant that contains molecules with intrinsic bioactivities on animal physiology and metabolism and possesses intense antimicrobial (Dorman and Deans, 2000; Ultee et al., 2002; Venkitanarayanan et al., 2013), anticoccidial (Bozkurt et al., 2016), antifungal (Daouk et al., 1995), and antioxidant properties (Yanishlieva et al., 1999; Cervato et al., 2000). These activities are mainly attributed to its main components, the phenol monoterpenes carvacrol and thymol (**THY**). Carvacrol and THY are both given a generally recognized as safe status by the Food and Drug Administration (Burdock, 2010), implying that their responsible use is safe. THY has demonstrated either non neurotoxic effects or even neuroprotective effects in cortical neurons (Garcia et al., 2006; Delgado-Marín et al., 2017). As an animal feed supplement, THY has been shown in mammals (rats) to help maintain higher levels of polyunsaturated fatty acids in the liver, brain, kidney, and heart (Youdim and Deans, 1999a,b and 2000). These results suggest a good THY activity as an effective free radical scavenger influencing the in vivo antioxidant defense system. In birds, dietary supplementation with thymol has also shown to improve the oxidative stability of chicken eggs (Botsoglou et al., 1997) and meat during storage (Luna et al., 2010). Due to the fact that the oxidative status of any system is the result of the balance between pro-oxidants and antioxidants (Smet et al., 2006), high relevance has to be assigned to oxidative features of animal feed. However, no recent study has been observed regarding poultry feed oxidative stability consequences of THY supplementation. The aim of this study was therefore to determine whether the inclusion of THY in a poultry feed mash can modulate the oxidative/degradative processes along storage, as well as induce changes in its fatty acids composition. Treatment with the widely used antioxidant BHT was used as a positive control.

MATERIAL AND METHODS

The experiment was carried out using a pelleted grower feed (Table 1). Thymol (THY, SAFC®, ≥99%, FCC, USA) and butylated hydroxytoluene (BHT, Fluka AG, Buchs SG, Switzerland) were used as feed additives. Fresh feed was assigned to 1 of 4 treatments: control (CON, no additive), Vehicle (VEH, ethanol 96%), 400 mg BHT/kg feed and 400 mg THY/kg feed. Three independent replicates of each treatment were taken and individually stored at 23 ± 3°C and 40 ± 5% relative humidity along 0, 30, and 60 d. Samples of each group were taken at each period of storage and stored at –20°C until further analysis.

Table 1. Composition (%) of experimental basal diets where antioxidants additives are added.

	Grower (3–5 weeks)
Corn	62.05
Pelleted soy	17.04
Soybean meal	12.50
Soybean oil	0.50
Animal meal	3.36
Limestone powder	0.62
Salt	0.31
DL methionine	0.38
Sodium bisulfate/butirate	0.20
Vitamin premix[1]	0.10
Lysine HCl	0.21
Choline chloride	0.07
Mineral premix[2]	0.10
Antioxidant premix	0
Calculated to contain	
Crude protein, %	21.50
ME, kcal/kg	3150
Calcium %	0.90
Available phosphorus	0.35

[1]Vitamin premix provided the following amounts per kilogram of diet: vitamin D3, 200 IU; vitamin A, 1.500 IU; vitamin E, 101 IU; niacin, 35 mg; D-Pantothenic acid, 14 mg; riboflavin, 4.5 mg; pyridoxine, 3.5 mg; menadione, 2 mg; folic acid, 0.55 mg; thiamine, 1.8 mg.

[2]Mineral premix provided the following amounts per kilogram of diet: Mn, 11.0%; Zn, 11.0%; Fe, 6.0 %; I, 2.0 ppm; Mg, 2.68%; Se, 600 ppm.

Lipid Extraction

Peroxide value (**PV**), acidity, and fatty acid composition are normally performed on liquid samples. As poultry feeds consist of solid matrix, the addition of an extraction step in the procedure was required. Thus, 25 g of minced feed was macerated with 50 mL of hexane in darkness during 24 h. Hence, neutral and nonpolar lipids were the main components analyzed in this study. Extracts were filtered and evaporated (25°C). Analytical degree chemicals were used in this study.

Peroxide Value (PV)

Peroxide value was tested using the American Oil Chemist's Society official method (AOAC Cd 8-53, 1993) slightly modified. Briefly, after evaporation, fat extract obtained were weighted and equally divided in 3 × 15 mL transparent vials. Residues were dissolved in 4 mL of a 3:2 acetic acid: chloroform solution and swirled until dissolution. Three aliquots were separately titrated as follows: 0.5 mL of saturated KI was added and immediately swirled. The solution was reposed with privation of light during exactly 1 min. Then, 5 mL of distilled water was added. The solution was immediately titrated with a standardized 0.001 M sodium thiosulfate solution with constant agitation until the yellow color vanished. After adding 0.5 mL of starch indicator solution, titration was continued until the blue color disappeared. PV was calculated as follows:

(1) PV (milliequiv peroxide/kg fat) = (S – B) × M × 1,000/ME (g)

Where:

B: volume of titrant, mL of blank (titration without sample)
S: volume of titrant, mL of sample
M: molarity of sodium thiosulfate solution
ME: mass of fat (g) (approx. 0.5 g)

Titratable Acidity

Titratable acidity (**TA**), or acidity, was determined according to a reference methodology (ISO 1740:2004 (IDF 6: 2004)) and slightly modified in order to be applied for poultry feed mash. Briefly, after evaporation, fat extracted (from 25 g feed) was weighted and the residue was dissolved in 14 mL of denaturized ethanol (1:10 methanol: ethanol). Then, 14 mL of diethyl ether were added and swirled. Three 7 mL aliquots were transferred into 3 125-mL Erlenmeyer flasks. Phenolphthalein was added (0.3 mL each) and solutions were immediately titrated with standardized 0.02 M potassium hydroxide with constant agitation until the pink color appeared (stable and persistent). Acidity expressed as % w/w oleic acid (molecular weight = 282.5 g/mol) was obtained as follows:

(2) TA (% w/w oleic acid) = $[(S - B) \times M_{KOH} \times 282.5 \times 0.4]/ME$ (g)

Where:

B: average volume of titrant, mL of blank (titration without sample)
S: average volume of titrant, mL of sample
M_{KOH}: molarity of potassium hydroxide
ME: mass of fat (g) (approx. 0.5 g)

Fatty Acid Relative Composition

Lipids extracted (approx. 0.4 g) were derived to methyl esters and analyzed by gas chromatography (**GC**) according to Luna et al. (2012). After alkaline saponification (1 M potassium hydroxide in methanol), the unsaponifiable matter were extracted with n-hexane and discarded. The fatty acid methyl esters (**FAMEs**) were obtained using 1 M sulfuric acid in methanol and analyzed by gas chromatography. Standards and mass couple to gas chromatography were used to identify each compound. FAMEs were analyzed by gas chromatography on a 30 m fused capillary column with an internal diameter of 0.25 mm (Polyethylene Glycol, Perkin Elmer Elite-WAX). The analysis was performed on a Perkin-Elmer Clarus® 500 Gas Chromatographer equipped with a flame ionization detector. Helium was used as carrier (constant flow of 49.6 psi). The injection port temperature was 250°C and the detector temperature was 250°C. Oven program temperature started on 180°C for 5 min and increased to 200°C at 4°C/min, and held at that temperature for 5 min. After that, the temperature was increased to 230°C at 3°C/min and held there for 25 min. Quantification was carried out by normalization and relative area percentages of each FAME detected. The analysis in this study includes the 5 fatty acids that were found in higher proportion (about 98% of the total areas determined). All determinations were conducted in duplicate for each sample.

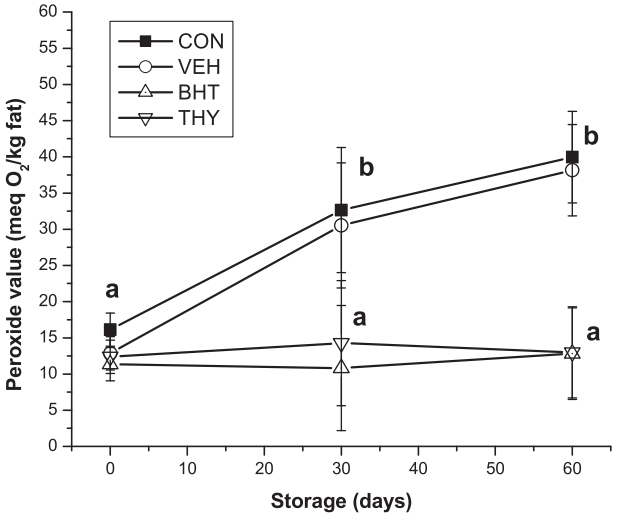

Figure 1. Effect of storage on peroxide value, mean (±SE) of poultry feed samples of control (CON), vehicle (VEH), butyl hydroxytoluene (BHT), and thymol (THY) evaluated after 0, 30, and 60 d of storage at 23 ± 3°C. [a,b]Values not sharing a common letter differ statistically at $P < 0.05$.

Statistical Analysis

Statistical analyses were performed using the Infostat statistical software package (Di Rienzo et al., 2016). Data were analyzed using a general linear mixed model evaluating feed supplementation (4 levels) and storage time (3 levels) as fixed effects and replica identity as a random variable (to include the "repeated sampling" effect into the analysis). Fisher's least significant difference tests were used for post-hoc comparisons of means. The homogeneity of the variances was tested. A probability level of less than or equal to 0.05 was considered to represent significant differences, and statistical trends (or tendency to a difference) were reported when $0.05 < P < 0.15$.

RESULTS

The extent of lipid peroxidation of feed (as PV) as a function of storage time and feed additive is illustrated in Figure 1. As expected, storage for 30 and 60 d increased the levels of PV ($F_{3,8} = 18.00$; $P = 0.001$). Interestingly, the concentration of lipid peroxides (PV) was influenced by feed additive treatment ($F_{2,16} = 23.92$; $P = 0.001$) and an interaction between storage time and feed additive was also found ($F_{6,16} = 5.14$; $P = 0.01$). At 0 d of storage (non-stored samples), post-hoc analysis

Table 2. Mean (±SE) titratable acidity (TA) expressed as grams of oleic acid/100 g fat of extruded samples of control (CON), vehicle (VEH), butyl hydroxytoluene (BHT), and thymol (THY) groups.

Diet	Storage time (d)		
Treatment	0	30	60
CON	17.31 ± 3.12	21.36 ± 3.74	46.11 ± 5.44
VEH	18.93 ± 3.12	18.14 ± 3.74	40.20 ± 5.44
BHT	21.48 ± 3.12	25.5 ± 3.74	42.26 ± 5.44
THY	20.58 ± 3.12	22.38 ± 3.74	45.07 ± 5.44

showed no differences in the concentration of peroxides between the different feed treatment. However, after 30 d and, particularly, 60 d of storage, increased ($P < 0.05$) PV concentrations were detected in CON and VEH in comparison to the feed supplemented with BHT and THY.

Results from TA (g of oleic acid/100 g fat) in feeds from different groups are shown in Table 2. As was expected, storage for 30 or 60 d increased the levels of TA in those samples ($F_{2,16} = 15.15$; $P = 0.001$). However, neither effects of feed additive nor an interaction between the additive and storage time were detected on this variable ($P > 0.86$ in both cases).

The FA composition in each additive treatment sample at 0, 30, and 60 d of storage is shown in Table 3. A summary of statistics for all variables is provided in Table 4. Changes in palmitic, oleic, linoleic, saturated, polyunsaturated and PUFA/SFA relative composition were found with storage time ($P < 0.01$ in all cases). Interactions or trends towards interactions between storage time and treatment were found for linoleic acid (18:2) ($P = 0.001$), saturated FA ($P = 0.12$), PUFA ($P = 0.01$) and PUFA/SFA ($P = 0.07$). Post-hoc analysis showed increases ($P < 0.05$) in the saturated FA value with storage time both in CON and VEH feed samples compared to THY and BHT feed samples that did not change with storage time. On the other hand, decreases ($P < 0.05$) in PUFA and PUFA/SFA were found with storage time both in CON and VEH feed samples compared with the THY and BHT samples. Again, no changes with storage time were found in THY and BHT samples. While decreased linoleic acid values ($P < 0.05$) were found with storage time both in CON and VEH feed samples compared with the THY samples, BHT samples showed intermediate values that were not different from CON, VEH, or THY treated samples. No treatment or storage time effects were detected for stearic acid (18:0).

DISCUSSION

This research shows increased lipid peroxidation in poultry feeds stored for 30 and 60 d. The observed oxidation was similarly delayed by the addition of THY and the synthetic antioxidant BHT. To our knowledge, this is the first study suggesting an oxidative protective effect of THY on poultry feed as well as the usefulness

Table 3. Fatty acid composition of feed samples of Control (CON), vehicle (VEH), BHT, and thymol (THY) groups at 0, 30, and 60 d of storage.

	CON			VEH			BHT			THY		
Composition	0	30	60	0	30	60	0	30	60	0	30	60
Palmitic acid (16:0)	12.9 ± 0.8	15.8 ± 1.0	17.8 ± 0.9	13.3 ± 0.8	16.5 ± 1.0	17.6 ± 0.9	13.7 ± 0.8	15.1 ± 1.0	14.9 ± 0.9	13.7 ± 0.8	14.1 ± 1.0	14.7 ± 0.9
Stearic acid (18:0)	2.71 ± 0.3	3.14 ± 0.3	3.59 ± 0.3	3.31 ± 0.3	3.75 ± 0.3	3.80 ± 0.3	3.39 ± 0.3	3.27 ± 0.3	3.67 ± 0.3	3.54 ± 0.3	3.28 ± 0.3	3.07 ± 0.3
Oleic acid (18:1)	26.0 ± 0.4	24.1 ± 0.6	24.6 ± 1.0	26.1 ± 0.4	23.4 ± 0.6	24.6 ± 1.0	26.1 ± 0.4	24.8 ± 0.6	25.1 ± 1.0	26.1 ± 0.4	24.8 ± 0.6	24.5 ± 1.0
Linoleic acid (18:2)	54.5 ± 0.6^{a}	53.5 ± 0.5^{a,b}	50.9 ± 1.0^{c}	54.0 ± 0.6^{a}	53.7 ± 0.5^{a}	51.6 ± 1.0^{b,c}	53.4 ± 0.6^{a,b}	52.5 ± 0.5^{a,b}	52.9 ± 1.0^{a,b}	53.1 ± 0.6^{a,b}	54.4 ± 0.5^{a}	54.3 ± 1.0^{a}
Linolenic acid (18:3)	3.91 ± 0.4	3.40 ± 0.1	3.13 ± 0.3	3.33 ± 0.4	2.60 ± 0.1	2.45 ± 0.3	3.47 ± 0.4	4.31 ± 0.1	3.43 ± 0.3	3.54 ± 0.4	3.34 ± 0.1	3.42 ± 0.3
Saturated (SFA)	15.6 ± 0.8^{d}	19.0 ± 1.1^{a,b,c}	21.4 ± 1.0^{a}	16.6 ± 0.8^{c,d}	20.3 ± 1.1^{a,b}	21.4 ± 1.0^{a}	17.1 ± 0.8^{c,d}	18.3 ± 1.1^{b,c,d}	18.5 ± 1.0^{b,c}	17.2 ± 0.8^{c,d}	17.4 ± 1.1^{c,d}	17.8 ± 1.0^{b,c,d}
Polyunsaturated FA (PUFA)	58.4 ± 0.6^{a}	56.9 ± 0.6^{a}	54.0 ± 1.1^{b}	57.3 ± 0.6^{a}	56.3 ± 0.6^{a,b}	54.1 ± 1.1^{b}	56.8 ± 0.6^{a}	56.8 ± 0.6^{a}	56.4 ± 1.1^{a}	56.7 ± 0.6^{a}	57.8 ± 0.6^{a}	57.7 ± 1.1^{a}
PUFA/SFA	3.80 ± 0.2^{a}	3.07 ± 0.2^{b,c,d}	2.53 ± 0.2^{d}	3.45 ± 0.2^{a,b}	2.79 ± 0.2^{c,d}	2.54 ± 0.2^{d}	3.33 ± 0.2^{a,b,c}	3.10 ± 0.2^{b,c}	3.09 ± 0.2^{b,c}	3.31 ± 0.2^{a,b,c}	3.33 ± 0.2^{a,b,c}	3.26 ± 0.2^{a,b,c}

[a-d]Means within rows with different letters differ at $P < 0.05$.

Table 4. Summary of fatty acid statistical results on feed samples of Control (CON), vehicle (VEH), BHT and thymol (THY) groups at 0, 30, and 60 d of storage.

	Feed additive treatment			Storage time			Interaction Treatment × Storage		
Effects	DF	*F*-value	*P*-value	DF	*F*-value	*P*-value	DF	*F*- value	*P*-value
Palmitic acid (16:0)	3, 8	2.18	0.17	2, 16	10.10	0.00	6, 16	1.33	0.30
Stearic acid (18:0)	3, 8	1.28	0.35	2, 16	1.18	0.33	6, 16	1.26	0.33
Oleic acid (18:1)	3, 8	0.56	0.65	2, 16	6.30	0.01	6, 16	0.27	0.94
Linoleic acid (18:2)	3, 8	0.58	0.64	2, 16	8.79	0.00	6, 16	5.66	0.00
Linolenic acid (18:3)	3, 8	4.83	0.06	2, 16	2.53	0.11	6, 16	1.63	0.20
Saturated FA (SFA)	3, 8	2.02	0.19	2, 16	12.23	0.00	6, 16	2.05	0.12
Polyunsaturated FA (PUFA)	3, 8	0.97	0.45	2, 16	9.30	0.00	6, 16	4.16	0.01
PUFA/SFA	3, 8	1.21	0.37	2, 16	11.34	0.00	6, 16	2.46	0.07

of PV as a fast and sensitive simple procedure to detect changes in poultry feed oxidative stability.

The observed increase in PV is evidence that feed storage has increased the lipid oxidation in feed mashes both in the CON and VEH groups, and revealed that the presence of antioxidants as BHT and THY can delay or counteract this process up to at least 60 d. These results are in agreement with previous studies showing a high antioxidant activity of THY (Yanishlieva et al., 1999; Lee et al., 2004, 2005; Reynertson et al., 2008; Beena and Rawat, 2013). This study supports other reports showing an improvement in the oxidative stability of poultry products as consequence of dietary supplementation of THY (Botsoglou et al., 1997; Luna et al., 2010, 2012). In treatments with no antioxidant supplementation, the oxidative process increased PV to significant higher values in both 30 and 60 d of storage CON and VEH feed samples. The prevention of oxidative deterioration induced in both THY and BHT supplemented feeds is consistent with results reported by Luna et al. (2010), where similar antioxidant activity were also found in stored poultry meat after dietary supplementation with BHT or THY. Al-Malki (2010) compare the antioxidant and liver protective effects of BHT and THY, informing beneficial effects on both damaged and normal hepatocytes. Both compounds were reported to prevent lipid peroxidation and improve antioxidant enzymes activities. In this line, Fernandez et al. (2015) reported increases in polyunsaturated fatty acids in egg yolks from hens fed with 6.25 g thymol/kg of feed. The study is consistent with the hypothesis that an improvement on the oxidative stability of the feed is directly related with effects on the animals consuming it. Accordingly, McGill et al. (2011) showed in broilers that supplementation with antioxidants can help to counteract the deleterious effect of high PV diets on live performance parameters, feed conversion and immunity. Our results suggest the usefulness of PV as a potential and simple methodology to assess oxidative deterioration of feeds, complementing those of Smet et al. (2006), where the FRAP and DPPH methods are employed to evaluate antioxidant potential in feed stuffs.

Broiler feedstuffs always include fat components that are provided by raw materials, and also by added fats. Soybean oil was the fat component added in this study. Fat components tend to break down during storage and deterioration may be also accelerated by insect attack, microorganisms and/or hydrolytic enzymes (Chow, 1980). This deterioration is observed as a break of glycerides and causes an increase in free fatty acids which may generate off-flavors related to oxidation of fats. The increase of free fatty acids as a result of lipid breakdown may therefore be of particular relevance regarding the quality of the feed during storage (Chow, 1980). The current study did not show effects of feed additives on TA. However, as expected, significant TA increases were found with time of storage consistently with the studies by Chow (1980) and Ramezanzadeh et al. (1999) reporting that fats tend to break down during storage, increasing free fatty acids and hydrolytic rancidity. Future studies with increased temperature and humidity conditions would be worthy to determine whether the THY or BHT are able to reduce the negative TA changes when feed mash are stored under more extreme conditions.

As it was observed in TA, relative fatty acid composition present differences related to storage time, reflecting the influence of long time storage on chemical characteristics of poultry feeds. Feed additive treatment in combination with storage time showed an increase in the saturated FA value both in CON and VEH feed samples compared to THY and BHT feed samples that did not change with storage time. Consistently, decreases in PUFA and PUFA/SFA ratio were also found with storage time both in CON and VEH feed samples compared with the THY and BHT samples. Again, no changes with storage time were found in THY and BHT samples. These findings suggest an antioxidant protection of the latter against chemical spoilage of feed mash. This study complement that of Njobeh et al. (2006), who reported increased free fatty acids and peroxide values in broiler feeds stored during 60 d, and highlight the need for using preservatives to reduce or counteract feed deterioration during feed storage. Several studies inform antioxidant activity of essential oils containing thymol (Lee et al., 2004; Brenes and Roura, 2010; Zeng et al., 2015), with different ways of incorporation to the feed. Considering that oregano essential oil contains aprox. 30% of

thymol, and that oregano leaves contain approx. 0.1% of essential oil, the dose of thymol applied per kg of feed in the current study represents approximately 650 g of oregano leaves, providing a strong antioxidant effect with no significant contribution on other main feedstuff components (crude protein, carbohydrates, lipids, etc).

In conclusion, the absence of PV increase with time in the THY- and BHT-treated groups, as well as their fatty acids profiles are consistent with similar THY and BHT protective effects on feed mash lipid oxidation along 60 d of feed storage under standard conditions. Nevertheless, studies with different levels and types of added fats would be worthy to further prove the proposed delay in the oxidative deterioration of stored feeds. Our findings suggest the potential usefulness of THY as a natural alternative to help sustain quality of poultry feed, and complement studies evaluating physiological and/or productive consequences of THY feed supplementation on animal production.

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